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Analysis of binary mixtures of cephalothin and cefoxitin by using first-derivative spectrophotometry

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Abstract

A method for the analysis of two-component mixtures of cephalothin and cefoxitin using zero-crossing first-derivative spectrophotometry is described. This technique permits the quantification of these drugs with closely overlapping spectral bands without any separation step. Linear calibration graphs of first-derivative values at 235.00 and 236.75 nm for cephalothin and cefoxitin, respectively, with negligible intercepts were obtained versus concentration in the range 4.0–32.0 $\mu g\,ml^{-1}$ for both antibiotics. This paper presents a systematic examination of the experimental data by applying an exhaustive statistical analysis to demonstrate the validity of the method. The results of the determination of these antibiotics in mixtures of injectable dosage forms are also presented, together with their determinations in physiological serum and glucosed physiological serum.

Keywords: Cephalothin; Cefoxitin; Derivative spectrophotometry; Simultaneous determination; Pharmaceutical dosage forms

1. Introduction

Cephalothin is a semisynthetic cephalosporin antibiotic. Based on its spectrum of activity, cephalothin is classified as a first-generation cephalosporin. Like other first-generation cephalosporins, this antibiotic is active against many Gram-positive aerobic cocci but has limited activity against Gram-negative bacteria [1]. Cefoxitin is a semisynthetic cephamycin antibiotic [2]. This drug is a β-lactam antibiotic structurally and pharmacologically related to cephalosporins. The cephamycins contain a methoxy group rather than a hydrogen at the 7α-position on the β-lactam ring of the cephalosporin nucleus. The 7-methoxylated cephalosporins possess marked resistance to the action of β-lactamases from Gram-positive and Gram-negative organisms [3]. Of the Gram-negative microorganisms generally susceptible to cephalothin, i.e. *E. coli*, *P. mirabilis* and *Klebsiella*, cefoxitin was significantly more active than cephalothin. The Gram-positive microorganisms, on the other hand, were less susceptible to cefoxitin than to cephalothin [2,4].

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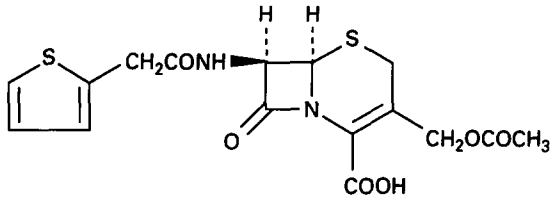
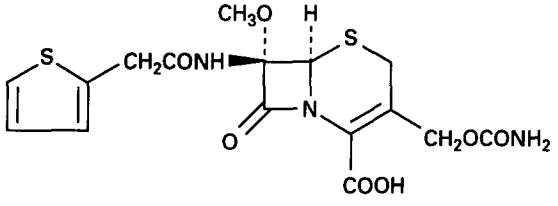


CEPHALOTHIN



CEFOXITIN

Cephalothin and cefoxitin have very similar chemical structures and so their absorption spectra show closely overlapping bands. Derivative spectrophotometry presents greater selectivity than does normal spectrophotometry and so offers a convenient solution to the problem of resolving spectral overlap in the analysis of multicomponent systems [5,6]. Peak-to-peak and baseline measurements (generally referred to as graphical measurements) and zero-crossing measurements are the most common techniques used to prepare analytical working curves. The zero-crossing method involves the measurement of the absolute value of the total derivative spectrum at an abscissa value (wavelength) corresponding to the zero-crossing point of the derivative spectrum of the interfering component. At this wavelength, the amplitude of the derivative signal of one of the two components passes through zero; measurement of the value of the derivative spectrum of a mixture, made at the zero-crossing point of the derivative spectrum of one of the components, is therefore a function only of the concentration of the other component [7].

O'Haver [8] discussed the potential of derivative spectrophotometry in clinical chemistry. Fell [9] has demonstrated the possibilities offered by this technique for the analysis of pharmaceutical formulations.

Different colorimetric methods have been reported for the assay of cephalosporins using either ammonium vanadate [10], ninhydrin [11] or ammonium molybdate in acidic media [12]. The official method for the determination of cephalosporins was based on iodometric titration [13]. These methods are not specific. Derivative spectrophotometry has been employed for the simultaneous determination of substances of clinical interest, including cephalosporins, in mixtures, e.g. cephapirin and cefuroxime [14], 7-aminocephalosporanic acid and 7-aminodesacetoxycephalosporanic acid [15].

The aim of this work was to demonstrate the ease with which the proposed derivative method circumvents the problem of overlapping spectral bands, allowing the simultaneous determination of these antibiotics in mixtures without the need for prior separation. The method was applied to mixtures of these drugs in both pure forms and pharmaceutical formulations. Satisfactory sensitivity, accuracy and precision were noted. Other advantages of the method are its simplicity and speed.

2. Experimental

2.1. Reagents

All experiments were performed with analytical-reagent grade chemicals and water purified using a Milli-Q system (Millipore).

Stock cephalothin solution, 100 μg ml^{-1}, was prepared by dissolving the standard sodium salt of cephalothin (Sigma Chemical Company Products) in water.

Stock cefoxitin solution, 100 μg ml^{-1}, was prepared by dissolving the standard sodium salt of cefoxitin (Sigma Chemical Company Products) in water.

Buffer solution, 0.05 M, of pH 7.2 was prepared by mixing sodium dihydrogenphosphate (0.1 M) with sodium hydroxide (0.1 M) of analytical-reagent grade.

The stock solutions were stored below 5°C in the dark. Working standard solutions of the desired concentration were obtained by diluting the stock solutions with water as required.

Injectable dosage forms of Keflin Neutro (Lilly) were stated to contain 1 g of cephalothin sodium salt per vial. Intravenous injectable dosage forms of Cefaxicina (Compañía Española de la Penicilina y Antibióticos), were stated to contain 1 g of cefoxitin sodium salt per vial. Intravenous and intramuscular injectable dosage forms of Mefoxitin (Merck Sharp & Dohme de España) were stated to contain 1 and 0.5 g of cefoxitin sodium salt per vial respectively.

Saline physiological sera (0.9% sodium chloride) were obtained from Apiroserum (Instituto de Biología y Sueroterapia) and Grifols (Laboratorios Grifols). Glucosed physiological serum (5% anhydrous glucose (dextrose)) was obtained from Apiroserum (Instituto de Biología y Sueroterapia).

2.2. Apparatus

All spectral measurements and treatment of data were carried out with a Beckman Instruments DU-70 spectrophotometer connected to an IBM-PS computer with Beckman Data Leader software [16] and an Epson FX-850 printer. Among other possibilities, this system provides the capability for data manipulation for smoothing and derivatives. Moreover, special Zoom In/Out and Trace functions allow data details and interpolation values to be analysed.

2.3. Sample preparation and procedure

In 25 ml calibrated flasks, suitable aliquots of a standard solution of cephalothin or cefoxitin or their binary mixture were transferred to produce working solutions in the range 4.0–32.0 $\mu g\,ml^{-1}$ of both antibiotics. Then, 5.0 ml of buffer solution (pH 7.2) were added to each of the calibrated flasks and diluted to volume with distilled water.

The absorption spectra were recorded against a reagent blank (the same as the samples without the compounds to be determined) using a 1.0 cm quartz cell and stored in an IBM-PS computer. The first derivative was then calculated. The absolute values of the first derivative were measured at 235.00 and 236.75 nm and, by using an appropriate calibration graph, the cephalothin and cefoxitin concentrations, respectively, could be determined. Each calibration graph was constructed by varying the concentration of one antibiotic within the established range in the absence of the other compound.

2.4. Procedure for injections

Both cephalothin and cefoxitin powder for injection were used by dilution of the contents of proprietary vials of parenteral material in different media: Milli-Q water, physiological serum and glucosed physiological serum.

In aqueous medium, the contents of the vials of both antibiotics were placed in a 100 ml calibrated flask and dissolved in and diluted to volume with Milli-Q water. After prior dilution, the general procedure was applied to the resulting injectable solution.

For continuous intravenous infusion for clinical use, the contents of a 1 g vial of the drug (cephalothin or cefoxitin) may be dissolved in at least 10 ml of sterile water for injection and added to a compatible intravenous solution. Thus, for 500 ml intravenous infusions, the final dissolution has a concentration of 2 $mg\,ml^{-1}$. Hence the contents of the injectable vials containing cephalothin (Keflin Neutro, Lilly) and cefoxitin (Cefaxicina, Compañía Española de la Penicilina y Antibióticos; Mefoxitin, Merck Sharp & Dohme de España) were dissolved in the case of both saline physiological serum (0.9% sodium chloride) and glucosed physiological serum (5% anhydrous glucose), so that the concentration of both antibiotics used clinically was obtained. Subsequently, a suitable quantity of several sera containing both antibiotics, after prior dilution, was analysed following the general procedure described. The percentages of both antibiotics were calculated using the linear regression equations obtained for each pure compound.

3. Results and discussion

The stability of aqueous solutions of cephalothin and cefoxitin was studied by recording their absorption spectra. At first these spectra were measured every 30 min, and subsequently every day. No changes in the spectra were observed for at least 15 days when the solutions are stored at 5°C in the dark.

The changes in the absorption spectra with pH were studied between pH 2.0 and 12.5. The cephalothin and cefoxitin spectra remained unchanged in the pH range 2.0–9.0. A medium near to neutrality was considered suitable and pH 7.2 was chosen. The selected pH was provided by adding phosphate buffer solution of pH 7.2, in which the solutions of both compounds showed suitable stability.

Fig. 1 shows the absorption spectra of cephalothin (CL), cefoxitin (CX) and a mixture of both compounds (M). As can be seen, accurate absorption measurements of each antibiotic in a binary mixture appears to be impossible because of total overlap of the bands. The overlap of the spectra of cephalothin and cefoxitin results in the absence, in the zero-order spectrum of the mixture, of any spectral feature that can be used to assay the two drugs.

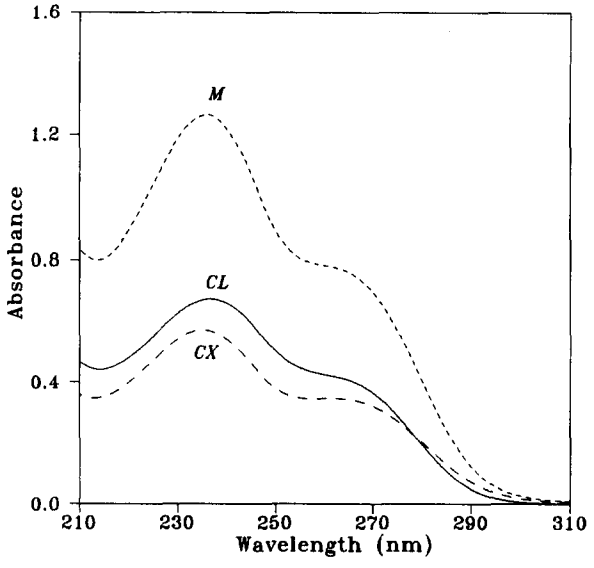


Fig. 1. Absorption spectra of (CL) cephalothin (20.0 μg ml^{-1}), (CX) cefoxitin (20.0 μg ml^{-1}) and (M) a mixture (20.0 μg ml^{-1} of each component); reference, reagent blank.

The traditional Vierordt method, which involves the use of two simultaneous equations, and the modified Vierordt method [17] provide results of poor accuracy and reproducibility when the absorption spectra of the components are not sufficiently separated. It was found that to resolve the problem of closely overlapping spectra, derivative spectrophotometry, particularly with digital processing, can be used. This technique involves the differentiation of a normal spectrum with respect to the wavelength. Savitzky and Golay [18] proposed a series of numerical tables for the smoothing of experimental data and for the computation of their derivatives. The main advantage of the method is that they obtain universal numerical functions, the convolution of which with the original vector data gives a smoothed vector and its successive derivative. Later, Steinier et al. [19] introduced a correction to Savitzky and Golay's reasoning and a check was performed by using a more general matrix formalism. Data Leader software has a smoothing algorithm based on the method of Savitzky and Golay, which can result in an improvement in the signal-to-noise ratio.

The zero-crossing method was used in this work with satisfactory results although the differential spectrophotometry showed reduced sensitivity because the sample absorbance bandwidth in this case was very wide compared with the wavelength separation of the absorbance peaks. Fig. 2 shows the first-derivative spectra of cephalothin (CL), cefoxitin (CX) and their mixture (M). The selected zero-crossing value of cephalothin appears at 236.75 nm, while the first derivative value of cefoxitin is zero at 235.00 nm. Other zero crossings of the two components appear at lower wavelengths, which are unsuitable because of the large interferent absorptions at wavelengths near 200 nm.

3.1. Selection of optimum instrumental conditions

The scan speed of the monochromator has vir-

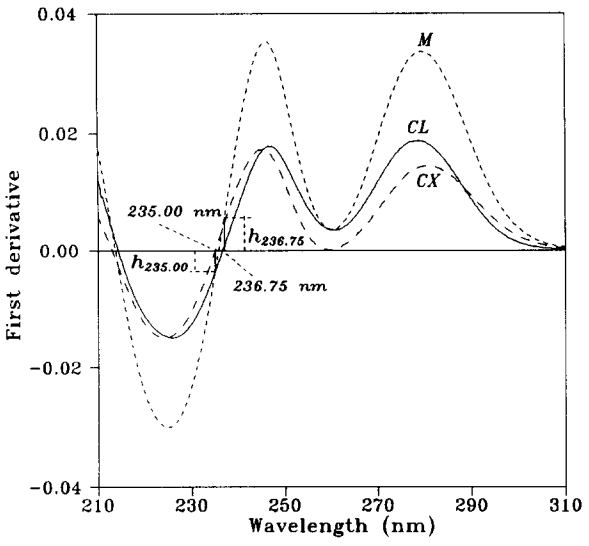


Fig. 2. First-derivative spectra of (CL) cephalothin (20.0 μg ml^{-1}), (CX) cefoxitin (20.0 μg ml^{-1}) and (M) a mixture (20.0 μg ml^{-1} of each component); reference, reagent blank.

tually no effect on the derivative signal obtained because the differentiation is obtained digitally. The scanning speed only determines the distance between data points which are collected every 0.05 s. A scan speed of 300 nm min^{-1} was selected, at which the instrument read 4.0 data points per nm, which is equivalent to 0.25 nm between readings.

The main instrumental parameter affecting the shape of the derivative spectra is the wavelength increment over which the derivatives are obtained ($\Delta\lambda$). This parameter needs to be optimized to give a well resolved large peak, i.e. to give good selectivity and higher sensitivity in the determination. Generally, the noise decreases with increase in $\Delta\lambda$, thus decreasing the fluctuation in a derivative spectrum. However, if the value of $\Delta\lambda$ is too large, the spectral intensity signal of first derivative deteriorates. Various values of $\Delta\lambda$ were tested and $\Delta\lambda = 8$ nm was chosen as the optimum in order to give an adequate signal-to-noise ratio. It was not necessary to use any smoothing function because of the low noise levels that the original and derivative spectra showed.

3.2. Calibration graphs and statistical analysis of experimental results

In order to test the mutual independence of the analytical signals of cephalothin and cefoxitin measured at 235.00 nm ($h_{235.00}$) and 236.75 nm ($h_{236.75}$), respectively, the following experiments were performed.

Four calibration graphs were constructed from the first-derivative signals by measuring at 235.00 nm for standard samples containing between 4.0 and 32.0 μg ml^{-1} of cephalothin, in absence of cefoxitin (p_0) and in presence of 8.0 (p_1), 20.0 (p_2) and 32.0 μg ml^{-1} (p_3) of cefoxitin. Fig. 3 exhibits two of these series of first-derivative spectra where the concentration of cephalothin was increased (from 4.0 to 32.0 μg ml^{-1}) in both cases and the first series (a) did not contain cefoxitin and the other series (b) contained a concentration of 20.0 μg ml^{-1} of cefoxitin. The experiments showed that the height at 235.00 nm ($h_{235.00}$) was proportional to the cephalothin concentration.

Similarly, four calibration graphs were prepared from the first-derivative signals by measuring at 236.75 nm for standard samples containing between 4.0 and 32.0 μg ml^{-1} of cefoxitin, in absence of cephalothin (q_0) and in the presence of 8.0 (q_1), 20.0 (q_2) and 32.0 μg ml^{-1} (q_3) of cephalothin. Fig. 4 exhibits two of these series of first-derivative spectra where the concentration of cefoxitin was increased (from 4.0 to 32.0 μg ml^{-1}) in both cases and the concentration of cephalothin was 20.0 μg ml^{-1} in (b) and the first series (a) did not contain cephalothin. Similarly, the experiments showed that the height at 236.75 nm ($h_{236.75}$) was proportional to the cefoxitin concentration.

In Fig. 3, all curves which contain the same concentration of cefoxitin converge to an abscissa value corresponding to the zero-crossing wavelength of cephalothin (236.75 nm). Similarly, in Fig. 4 all curves which contain the same concentration of cephalothin converge to an abscissa value selected to determine this antibiotic (235.00 nm) where the derivative signal of cefoxitin is zero.

Table 1 shows the results of the statistical analysis of the experimental data: the regression equations calculated from the calibration graphs, along with standard deviations of the slope and the intercept on the ordinate. The linearity of calibration graphs and the conformity of the systems to Beer's law are proved by the high values of the correlation coefficients of the regression equations.

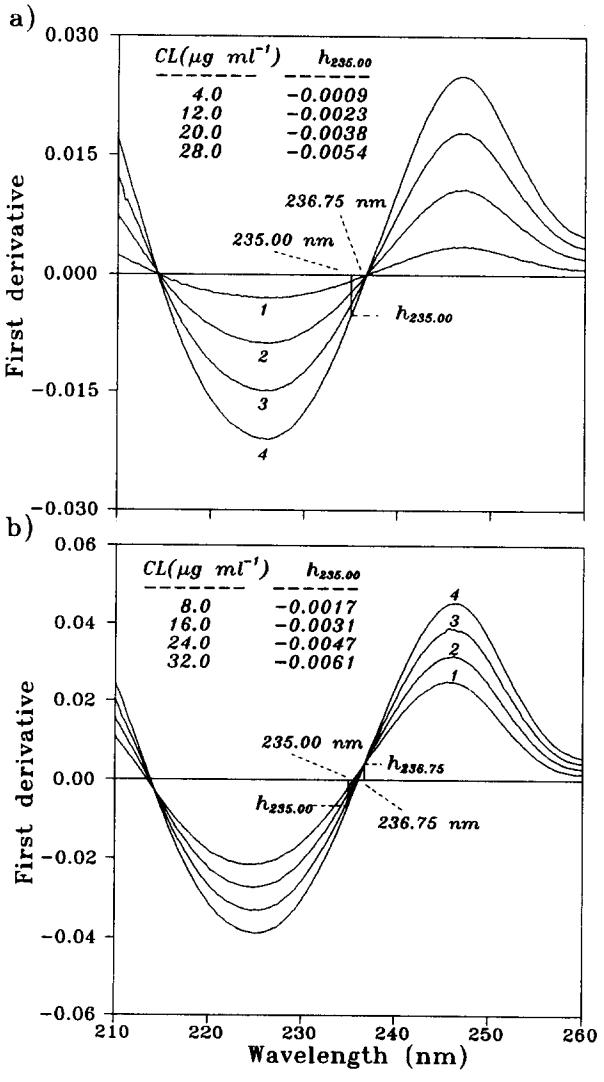


Fig. 3. Two series of first-derivative spectra of (a) concentrations of cephalothin varied from 4.0 to 32.0 $\mu g\,ml^{-1}$ and (b) 20.0 $\mu g\,ml^{-1}$ of cefoxitin, increasing the cephalothin concentration from 4.0 to 32.0 $\mu g\,ml^{-1}$.

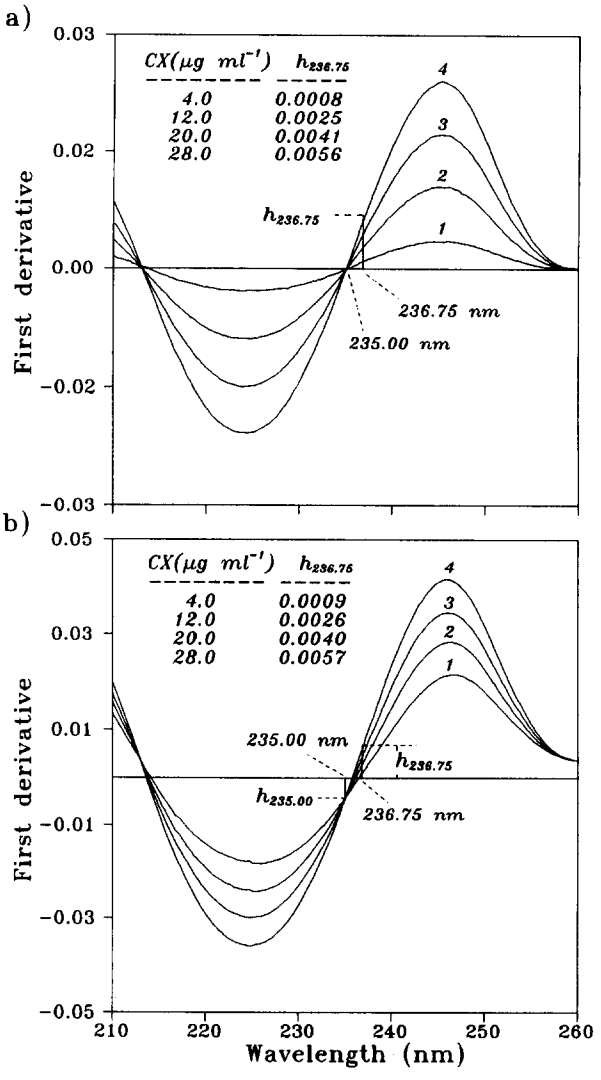


Fig. 4. Two series of first-derivative spectra of (a) concentration of cefoxitin varied from 4.0 to 32.0 $\mu g\,ml^{-1}$ and (b) 20.0 $\mu g\,ml^{-1}$ of cephalothin, increasing the cefoxitin concentration from 4.0 to 32.0 $\mu g\,ml^{-1}$.

The significance of the intercept on the ordinate of all regression lines was evaluated by applying the Student's t-test at 95% confidence level and seven degrees of freedom [20]. If the intercept on the ordinate for the regression lines calculated by the least-squares method is negligible, it is necessary to perform the fitting of the data again according to a function whose intercept on the

Table 1
Statistical analysis of calibration graphs in the determination of cephalothin (4.0–32.0 μg ml^{-1}) and cefoxitin (4.0–32.0 μg ml^{-1}) by first-derivative spectrophotometry for $n = 8$ standard specimens

Antibiotic determined	Other antibiotic present		Calibration graph	Slope ($\times 10^4$)	Intercept ($\times 10^4$)	Correlation coefficient	Standard deviation	
	Antibiotic	Concentration (μg ml^{-1})					Slope ($\times 10^5$)	Intercept ($\times 10^4$)
Cephalothin	—	—	p_0	1.89	0.79	0.999	0.27	0.54
($\lambda = 235.00$ nm)		8.0	p_1	1.92	0.89	0.999	0.22	0.45
	Cefoxitin	20.0	p_2	1.91	0.86	0.998	0.45	0.91
		32.0	p_3	1.93	0.61	0.999	0.41	0.83
Cefoxitin	—	—	q_0	1.96	1.18	0.999	0.25	0.51
($\lambda = 236.75$ nm)		8.0	q_1	1.98	0.32	0.999	0.31	0.62
	Cephalothin	20.0	q_2	1.96	1.18	0.999	0.40	0.81
		32.0	q_3	1.98	1.25	0.998	0.51	1.04

Table 2
Statistical parameters corresponding to the application of Student's t-test and evaluation of the residual error variance among the calibration graphs of cephalothin and cefoxitin by applying analysis of variance (95% level of confidence; 8 degrees of freedom)

Statistical parameters	Calibration graphs of cephalothin ($h_{235.00}$)				Calibration graphs of cefoxitin ($h_{236.75}$)			
	p_0	p_1	p_2	p_3	q_0	q_1	q_2	q_4
$t_{experimental}$	1.449	1.996	0.944	0.736	2.327	0.519	1.449	1.208
$t_{theoretical}$	2.365	2.365	2.365	2.365	2.365	2.365	2.365	2.365
m_0 ($\times 10^4$)	1.92	1.96	1.95	1.96	2.01	2.00	2.01	2.03
$F_{experimental}$			103				1.34	
$F_{theoretical}$			3.07				3.07	

Table 3
Resolution of synthetic mixtures of cephalothin and cefoxitin by first-derivative spectrophotometry

Theoretical concentration (μg ml^{-1})		[CL] ($h_{235.00}$)		[CX] ($h_{236.75}$)	
[CL]	[CX]	Pound (μg ml^{-1})	Recovery (%)	Pound (μg ml^{-1})	Recovery (%)
4.0	20.0	4.08	102.0	19.80	99.0
8.0	8.0	8.06	100.8	8.07	100.9
20.0	8.0	19.69	98.4	8.09	101.1
20.0	4.0	20.22	101.1	4.00	100.0
24.0	20.0	24.49	102.0	19.81	99.0
28.0	32.0	27.67	98.8	32.05	100.2
32.0	16.0	32.44	101.4	16.27	101.7
32.0	32.0	31.86	99.6	31.54	98.6

ordinate is zero and therefore the value of the slope (m_0) may be calculated. The results of this study for all the calibration graphs are reported in Table 2. It can be seen that the calculated t-values do not exceed the theoretical values and hence intercept on the ordinate is negligible in all instances. Consequently, the new values of the slope were calculated (Table 2).

The precision of the proposed method was evaluated by applying the statistical technique of analysis of variance, which enables the total variation to be broken down into its components, i.e. the variation between samples and the calibration graphs–sample interaction. The validity of the analysis of variance assumes that the residual error variance does not change from one sample to another or from one calibration graph to another.

To carry out an analysis of variance, the variance ratio ($F_{experimental}$) must be calculated and compared with the theoretical value of F [21] for adequate degrees of freedom at the 95% confidence level. Table 2 shows the results obtained. As can be seen, in both the cephalothin and cefoxitin calibration graphs the experimental value of F is smaller than theoretical value and so at the 95% confidence level the source of variation is not significant. Therefore, it can be deduced that the amplitude of the derivative signal of the mixture, measured at the zero-crossing point of the derivative spectrum of one of the two components, is a function only of the concentration of the other component, in accordance with the theoretical predictions, i.e. the variation of both $h_{235.00}$ and $h_{236.75}$ was not affected by the presence of cefoxitin and cephalothin, respectively, for any ratio of concentrations of the two components, over the full range tested.

The reproducibility for two different mixtures of the two antibiotics was tested. Replicate samples were measured ($n = 10$) containing 20.0 $\mu g\,ml^{-1}$ of each component; standard deviations of 0.46 and 0.48 $\mu g\,ml^{-1}$ were obtained for cephalothin and cefoxitin, respectively, and a relative error of 1.7% was found for each component, at the 95% confidence level. Also, replicate samples were measured ($n = 10$) containing 4.0 $\mu g\,ml^{-1}$ of each compound (concentration near the determination limits of each antibiotic); standard deviations of 0.15 and 0.16 $\mu g\,ml^{-1}$ and relative errors of 2.6 and 2.8% were obtained for cephalothin and cefoxitin, respectively, at the 95% confidence level.

The detection limits defined as the analyte concentration leading to a signal that is three times the blank standard deviation [22], were 1.00 and 0.97 $\mu g\,ml^{-1}$ for cephalothin and cefoxitin, respectively. The determination limits, defined as the analyte concentration leading to a signal that is ten times the blank standard deviation [23] were 3.35 and 3.23 $\mu g\,ml^{-1}$ for cephalothin and cefoxitin, respectively.

3.3. Applications

The validity of the proposed method was tested by successive determinations of cephalothin and cefoxitin in synthetic mixtures. The results are shown in Table 3. Satisfactory results were obtained for the recovery of both compounds, indicating that the method is effective for the simultaneous determination of cephalothin and cefoxitin.

Because of difficulties encountered in obtaining dosage forms containing simultaneously both of the antibiotics tested, the proposed method was applied to the determination of these drugs in mixtures of commercial injections. The assay was carried out as described under the procedure for injections when they are dissolved in an aqueous medium. Table 4 gives the results of these applications. Other components of the pharmaceutical preparations tested showed no absorption at the measured wavelengths.

Since cephalothin sodium and cefoxitin sodium are usually administered intravenously, the proposed method was applied to the determination of mixtures of the commercial injections in saline physiological serum (Apiroserum and Grifols, 0.9% sodium chloride) and glucosed physiological serum (Apiroserum, 5% glucose). Table 5 shows the results of five replicate analyses of mixtures of Keflin Neutro (cephalothin Na) with Cefaxicina and Mefoxitin (cefoxitin Na) in different media. The accuracy and precision are satisfactory. The different sera do not show interferences in the proposed method because their components do not absorb in the wavelength range of interest.

4. Conclusion

The simplicity, precision and selectivity of derivative spectrophotometry make it particularly

Table 4
Determination of cephalothin and cefoxitin in mixtures of commercial injections

Mixtures of pharmaceuticals		Cephalothin			Cefoxitin		
Cephalothin injection	Cefoxitin injection	Taken ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)	Recovery[b] (%)	Taken ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)	Recovery[b] (%)
Keflin Neutro	Cefaxicina	20.0	20.17	100.88 ± 1.88	20.0	19.56	97.80 ± 1.70
	Mefoxitin, 1 g	20.0	19.83	99.24 ± 1.25	20.0	19.76	98.76 ± 1.31
	Mefoxitin, 0.5 g	20.0	19.73	98.68 ± 1.53	20.0	20.05	100.20 ± 1.70

[a] Average of five determinations.
[b] Average of five determinations ± standard deviation.

Table 5
Results of the determination of cephalothin Na and cefoxitin Na in injection mixtures; Keflin Neutro, powder for injection, 1 g of cephalothin Na per injection; Cefaxicina, powder for injection, 1 g of cefoxitin Na per injection; Mefoxitin, powder for injection, 1 or 0.5 g of cefoxitin Na per injection

Serum sample	Mixtures of pharmaceutical products			
	1st derivative ($h_{235.0}$)		1st derivative ($h_{236.75}$)	
	Cephalothin injection	Mean result (%)[a]	Cefoxitin injection	Mean result (%)[a]
Physiological serum (Apiroserum) (0.9% sodium chloride)	Keflin Neutro	98.12 ± 1.53	Cefaxicina	97.80 ± 2.40
		102.52 ± 1.94	Mefoxitin	96.36 ± 1.31
Physiological serum (Grifols)	Keflin Neutro	99.76 ± 2.75	Cefaxicina	97.80 ± 2.40
		100.88 ± 1.48	Mefoxitin	97.32 ± 1.07
Glucosed physiological serum (Apiroserum) (5% glucose)	Keflin Neutro	100.34 ± 1.21	Cefaxicina	99.72 ± 2.01
		103.64 ± 2.47	Mefoxitin	98.28 ± 2.01

[a] Mean of five determinations ± standard deviation.

suitable for the simultaneous analysis of binary mixtures of compounds having overlapping spectra. In this work, the simultaneous determination of cephalothin and cefoxitin in binary mixtures was solved by applying the zero-crossing technique to the first-derivative spectra. The measurement wavelengths were 235.00 and 236.75 nm to determine the cephalothin and cefoxitin, respectively. The statistical analysis of the results indicated that the presence of one of the components does not interfere with the determination of the other.

The proposed method was successfully applied to the determination of the studied compounds in pharmaceutical dosage forms, in both aqueous medium and physiological serum (saline and glucosed). Satisfactory recoveries were found in all cases.

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